JOURNAL OF VIROLOGY, Jan. 1980, p. 411-422
0022-538X/80/01-0411/12$02.00/0

Vol. 33, No. 1

Vesicular Stomatitis Virus Growth in *Drosophila melanogaster* Cells: G Protein Deficiency

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In cultured *Drosophila melanogaster* cells, vesicular stomatitis virus (VSV) established a persistent, noncytopathic infection. No inhibition of host protein synthesis occurred even though all cells were initially infected. No defective interfering particles were detected, which would explain the establishment of the carrier state. In studies of the time course of viral protein synthesis in *Drosophila* cells, N, NS, and M viral polypeptides were readily detected within 1 h of infection. The yield of G protein and one of its precursors, G_1, was very low at any time of the virus cycle; the released viruses always contained four to five times less G than those produced by chicken embryo cells, whatever the VSV strain or serotype used for infection and whatever the *Drosophila* cell line used as host. Actinomycin D added to the cells before infection enhanced VSV growth up to eight times. G and G_1 synthesis increased much more than that of the other viral proteins when the cells were pretreated with the drug; nevertheless, the released viruses exhibited the same deficiency in G protein as the VSV released from untreated cells. Host cell control on both G-protein maturation process and synthesis at traduction level is discussed in relation to G biological properties.

Various serotypes of vesicular stomatitis virus (VSV) (6, 39), other rhabdoviruses such as Piry and Chandipura (7), and Fish rhabdoviruses (8) are able to multiply in *Drosophila melanogaster* or mosquitoes. Insects infected with these viruses are paralyzed after exposure to CO_2; this symptom is similar to that induced by sigma virus, a virus which is present in natural populations of *Drosophila* (29). Sindbis virus, an alphavirus which multiplies in mosquitoes and is transmitted by them to vertebrates, also develops in *Drosophila* (20, 35). These viruses induce almost no pathogenic effect in inoculated flies (for a review see G. Brun and N. Plus, *in* M. Ashburner and J. Thompson, ed., *The Genetics and Biology of Drosophila,* in press), which survive as the virus grows. A dynamic equilibrium is reached between weak continuous virus production and thermal inactivation of particles.

The study of virus growth in insects has been facilitated by the development of stable lines of cultured mosquito or *Drosophila* cells (17, 19, 36, 44). Various arboviruses have been multiplied in insect cell lines in vitro (3, 37, 40) and follow similar growth kinetics: after a lag time varying with the multiplicity of infection, the virus titer increases in the culture medium to a maximum, then falls to a constant level, which is maintained, giving a continuous virus release in the course of cell divisions (carrier state) (11, 34).

The mechanism by which the noncytopathic persistent state of infection is established in insect cells is unknown; interferon-like or antiviral activities have not been detected in infected mosquito cells (2, 10, 38), and defective interfering (DI) particles are not produced early in the alphavirus replication cycle in the same cells (16, 24).

The purpose of this paper is to describe virus growth and polypeptide synthesis in VSV-infected *Drosophila* cells with the object of understanding the virus control mechanism in insect cells.

It has been found that no blockage of host protein synthesis occurs when all the cells are infected; the cellular control which allows the virus production to decline to the carrier state level does not require production of DI particles or an important synthesis of one or more detectable cellular specific proteins. The VSV structural proteins (G, N, and M), its minor protein component (NS), and the partially glycosylated G precursor (G_1) are visible among the cell proteins; only the L chain is obscure. Nevertheless, the glycoprotein of the viral envelope is present only at low levels throughout the VSV replication cycle and in the released mature viruses. Experiments using actinomycin D show that

synthesis of the viral protein or mRNA was enhanced in treated *Drosophila* cells, especially in the case of G protein or G mRNA.

MATERIALS AND METHODS

Cells. The *Drosophila* cell line used in this study was line 75E7, except in the experiments in Table 2, where line 75B was also used. These virus-free lines (indexed by F. Hink *in Third Compilation of Insect Cell Lines,* in press) were isolated by F. Diatta and A. Ohanessian, respectively, in our laboratory according to the procedure already described (17). Cells were transferred at 4-day intervals by scraping and washing as many as possible off the surface of plastic flasks and diluted with Shields and Sang medium (43) plus 20% fetal calf serum. About 5×10^6 cells in 1 ml of medium were transferred in 35-mm-diameter plastic petri dishes. Cells were grown at 25°C for 36 h before infection (about 7.5×10^6 cells per dish).

Chicken embryo primary cell cultures and baby hamster kidney (BHK-21) cell monolayers, provided by F. Bussereau (Université Paris XI, Orsay, France), were used. These cells were grown in Eagle minimal essential medium supplemented with 6% calf serum and 10% tryptose.

Virus strains. VSV Indiana, BT78 (lot 1555), obtained from R. P. Hanson (Dept. of Veterinary Science, College of Agriculture and Life Sciences, University of Wisconsin, Madison) and described by F. Bussereau (6), was cloned two times on chicken embryo cells. In the experiments in Table 2, two other VSV strains were used: VSV New Jersey (Hazelhurst strain) (6), obtained from Dr. Hanson, and the large-plaque variant of VSV Indiana (VSV standard) obtained from J. Périès (Institut de Recherches sur les Leucémies, Hôpital St-Louis, Paris) (46).

Initial stocks of each virus were built up by infection of chicken embryo cell monolayers with clonally purified virus at a multiplicity lower than 0.01 PFU/cell to avoid production of interfering particles. After 48 h at 37°C, when most of the cells were lysed, the culture medium was collected and clarified by centrifugation at 6,000 rpm for 10 min in a Sorvall SS34 rotor. The supernatant, containing virus (2×10^9 to 4×10^9 PFU/ml), was distributed in 2-ml fractions and kept at −80°C.

Infection of *Drosophila* cell cultures. *Drosophila* cells (in a 35-mm petri dish) were infected 24 h after transfer. The culture medium was removed and replaced by 0.1 ml of the VSV suspension (2×10^9 PFU/ml). After adsorption for 1 h at 25°C, the inoculum was removed, cells were washed two times with 1 ml of TD (150 mM NaCl, 5 mM KCl, 0.7 mM disodium monophosphate, 0.8 mM Tris-hydrochloride, pH 7.6) plus 10% calf serum, and then 1 ml of Shields medium, supplemented with 10% and 20% fetal calf serum for lines 75B and 75E7, respectively, was added.

For VSV cycle studies, the noncumulative virus production by infected *Drosophila* cells was determined for each time of the cycle (one petri dish per point). Two hours before the released virus was collected, the culture medium was removed, the cells were washed two times with 1 ml of TD plus 10% calf serum, and then 1 ml of fresh Shields medium was added. After 2 h at 25°C, the culture medium containing the virus produced during this interval of time was collected and kept at −80°C until determination of the virus titer by plaque counting on chicken embryo monolayers.

Serial undiluted passage of VSV in *Drosophila*, BHK-21, and chicken embryo cells. To test the generation of VSV DI particles, *Drosophila,* BHK-21, or chicken embryo cells were infected with VSV (40 to 80 PFU/cell). The progeny virus was harvested 24 h after infection and transferred undiluted to fresh homologous cultures. The same process was repeated four times. Presence of DI particles after four passages was tested by interference in BHK-21 cells according to Holland et al. (22). BHK-21 cells were infected for 2 h with the last undiluted fourth stock, and then clonally purified VSV at 2×10^9 PFU/ml was added for 1 h. After removal and two washes of the excess virus, fresh Eagle minimal essential medium was added, and viral yield after 24 h at 37°C was determined by plaque counting on chicken embryo monolayers.

Radioisotopic labeling for analysis of infected cell proteins. The culture medium of the VSV-infected *Drosophila* or chicken embryo cells (in 35-mm petri dishes) was removed at the time indicated during the VSV cycle. After two successive washes with 1 ml of TD, 1 ml of TD was added plus ^{14}C-amino acids (5 μCi) and incubated for 30 min. The radioactive medium was then discarded, and cells were washed with cold TD (1 ml). All the following steps were performed at 4°C and in the presence of a serine protease inhibitor (1 mM), phenylmethylsulfonyl fluoride. Cells were scraped, collected in TD (1.5 ml final volume), and washed two times by centrifugation (5 min at 3,000 rpm) with 1 ml of TD and 1 ml of low-salt buffer (150 mM NaCl–10 mM Tris-hydrochloride, pH 7.5). The last cell pellet was suspended in the electrophoresis sample buffer (60 mM Tris-hydrochloride, pH 6.8; 3%, wt/vol, sodium dodecyl sulfate; 5%, vol/vol, 2-mercaptoethanol; 10%, vol/vol, glycerol; 0.01% bromophenol blue) and immediately heated at 95°C for 2 min.

Radioisotopic labeling and purification of VSV. [^{14}C]leucine-labeled viruses were grown in *Drosophila* cells (Falcon flask, 25 cm^2) as described above except that, after adsorption, the added Shields medium (3 ml) contained 1/20 standard leucine concentration, 2% fetal calf serum, and [^{14}C]leucine (5 μCi/ml). The supernatant of the culture was harvested 48 h after infection. [^{14}C]leucine-labeled viruses were also grown in chicken embryo cells as described for the preparation of the initial virus stock except that the medium (Eagle minimal essential medium) was diluted twofold, calf serum concentration was lowered to 2%, and [^{14}C]leucine (5 μCi/ml) was added after adsorption.

For virus purification, 3 ml of [^{14}C]leucine-labeled viruses was added to unlabeled VSV (2×10^{10} to 5×10^{10} PFU). Cell debris were removed by centrifugation (20 min at 6,000 rpm in a Sorvall SS34 rotor). Viruses were precipitated with polyethylene glycol according to McSharry and Benzinger (31); the virus pellet, dissolved in TD, was sonicated, laid on a 10 to 35% sucrose gradient (in 150 mM NaCl–10 mM Tris-hydrochloride, pH 8), and centrifuged for 30 min at 30,000 rpm in a Beckman SW41 rotor. About 20 fractions of

0.6 ml each were collected, and the virus peak was detected by its optical density at 260 nm; the virus-containing fractions were laid on a second gradient (20 to 70% sucrose in the same buffer) and centrifuged to equilibrium overnight at 35,000 rpm in a Beckman SW41 rotor. The virus-containing fractions were collected as before and kept at −80°C.

Polyacrylamide slab gel electrophoresis, autoradiography, and scanning. Polyacrylamide gel electrophoresis with sodium dodecyl sulfate was performed essentially according to the procedure described by Laemmli (26), except that a slab gel (1.2 mm thick) was used, the concentration of the electrophoresis buffer was raised twofold, and the gel contained a 5 to 13% linear gradient of acrylamide to obtain a better partition from the largest to the smallest proteins. Electrophoresis was run at room temperature for about 5 h at 20 mA (constant current) with bromophenol blue as the tracking dye. The slab gel was removed and stained with Coomassie brilliant blue, destained (48), dried, and exposed to Kodak Kodirex film. The exposed film was developed after 8 to 15 days; the absorbance of the film image was measured using the scanning densitometer accessory of an Isco U A-5 absorptiometer at 660 nm.

Materials. The Eagle minimal essential medium was purchased from Eurobio; calf serum came from Sorga; fetal calf serum was obtained from Gibco Biocult; polyethylene glycol (molecular weight, 6,000) was obtained from Touzart et Matignon; ^{14}C-amino acids (1.5 to 2 mCi/mg) and [^{14}C]leucine (250 to 300 mCi/mmol) were obtained from CEN Saclay (France); actinomycin and phenylmethylsulfonyl fluoride came from Merck; and sodium dodecyl sulfate, acrylamide, and *N,N'*-methylene bisacrylamide were obtained from Serva.

RESULTS

VSV growth after primary infection in *D. melanogaster* cells. *Drosophila* cells in culture were infected with VSV Indiana BT78 at an input multiplicity of 28 PFU/cell (infectivity was determined on chicken embryo primary cell culture). Since it has been shown in immunofluorescent studies (Ch. Richard-Molard, personal communication) that, in conditions identical to those used in this study, infection with more than 24 PFU/cell gave nearly 100% fluorescent cells 8 h after infection, it was possible to realize a one-step infection of all cells.

The VSV growth curve is shown in Fig. 1. The noncumulative virus production was determined during 2-h intervals. The lag time was less than 2h, the titer increased until about 8 h, reaching 1.2×10^8 PFU/ml per 2 h (16 PFU/cell per 2 h), and then decreased to a final level of about 2 PFU/cell per 2 h, about 20 h after infection, and remained approximately constant (carrier state). No cytopathic effect was observed.

Failure to detect DI particles of VSV during the first 24 h after infection. Since persistent infection of BHK-21 cells with VSV was demonstrated to require the continuous presence of DI particles (21), their presence in *Drosophila* cells could be responsible for the rapid lowering of the virus titer resulting in the carrier state. To test this hypothesis, we looked for the presence of DI particles during the first 48 h after infection with VSV. When the cells were infected in one step with VSV as described above, it was not possible to demonstrate a production of DI particles, as follows.

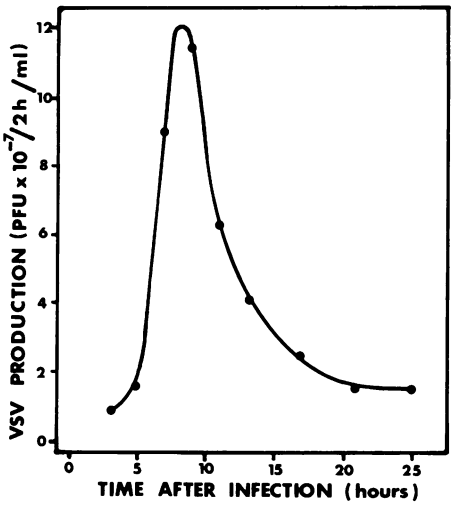

FIG. 1. *VSV growth curve in Drosophila cell culture. Drosophila cells, line 75E7 (one petri dish per point), were infected with VSV at an input multiplicity of 28 PFU/cell and processed as described in the text. Virus production during 2-h intervals was determined by plaque counting on chicken embryo monolayers.*

(i) The virion-containing supernatants collected between 1 and 8 h, 8 and 24 h, and 24 and 48 h after infection did not interfere with the replication of clonally purified VSV (Table 1) (the numbers found were not significantly different from the control).

(ii) The virus released during the first 24 h after infection did not reveal an extra peak or shoulder of DI particles, lighter than that of the normal virion, after centrifugation on velocity gradient (data not shown).

(iii) After four serial undiluted passages of VSV on *Drosophila* cells as described in Materials and Methods, no interference was observed (Table 1), whereas four passages under the same conditions on chicken embryo or BHK-21 cells revealed an interference of 96 and 99%, respectively, due to DI particle production.

(iv) Attempts were carried out to amplify on BHK-21 cells the DI particles which might have been produced by the infected *Drosophila* cells during the four serial passages. After purification, according to Doyle and Holland (15), a light DI particle shoulder (representing less than

TABLE 1. *Test for presence of DI particles during VSV cycle in Drosophila cells and in the fourth undiluted passage of VSV on various cells by interference in BHK-21 cells*[a]

Expt no.	Source of DI particles[b]	Inoculum[c] (PFU/cell)	24-h yield (PFU/ml)	Control[d] (PFU/ml)	Interference (%)
1	VSV released from *Drosophila* cells:				
	1-8 h	1.3	3.6×10^9	3.75×10^9	4
	8-24 h	0.7	3.7×10^9	3.75×10^9	2
	24-48 h	1.5	3.3×10^9	3.75×10^9	12
2	VSV obtained after four undiluted passages on:				
	Drosophila cells	1.5	1.8×10^9	2×10^9	10
	Chicken embryo cells	6.9	8.5×10^7	2×10^9	95.7
		1.5	4.9×10^8	6.6×10^9	92.5
	BHK-21 cells	1.6	7.1×10^6	2×10^9	99.6

[a] Mean values of two experiments.
[b] Virus stocks are described in the text.
[c] BHK-21 cells were infected for 2 h with the virion-containing supernatant to be tested for DI particle presence at the input multiplicity indicated. After removal of this first inoculum, clonally purified VSV was added at an input multiplicity of 20 PFU/cell for 1 h. The culture media were collected 24 h later, and virus titer was determined on chicken embryo monolayers.
[d] As control for each experiment, BHK-21 cells were infected only with clonally purified VSV (20 PFU/cell) for 1 h; the culture medium was collected 24 h later, and virus titer was determined.

10% of the normal virion peak) was detected, whereas similar experiments made with the fourth passage on chicken embryo or BHK-21 cells revealed broad DI particle peaks representing 70 and 200%, respectively, of the normal virion peak (results not shown).

Actinomycin D effect on VSV growth in *Drosophila* cells. Infection of vertebrate cells by VSV is cytopathic and leads to cellular lysis, whereas infection of insect cells results in persistent infection. The carrier state process may be regulated by some cell function, and actinomycin D may counteract this cellular control; this inhibitor has been described as enhancing virus titer in *Aedes albopictus* cells persistently infected by VSV (2), but not in freshly infected cells.

In *Drosophila* cells, a rise of the VSV titer was observed. The rise was maximal when actinomycin D reached a concentration of 0.1 μg/ml and was added 30 min before infection (results not shown). The growth curve of VSV in *Drosophila* cells that were pretreated in this way is shown in Fig. 2, compared to that of VSV in untreated cells and in chicken embryo fibroblasts. VSV production (expressed in PFU per cell) in pretreated cell culture was immediately enhanced up to eight times compared to that of untreated cells, yet it did not reach the high level obtained with chicken embryo cells grown at a higher temperature than insect cells.

This VSV growth curve is limited to 9 h, due to the drastic effect of the drug on the cells even when not infected, and although its concentration was very low. After 30 min of actinomycin D treatment, 80% of the uninfected cell RNA

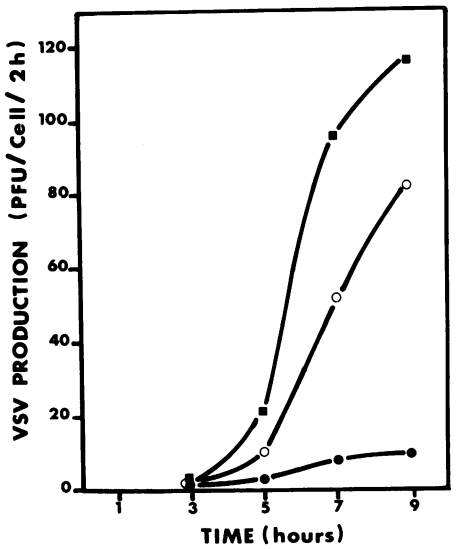

FIG. 2. *VSV growth curves in Drosophila cell cultures treated with actinomycin D and in chicken embryo cells. Drosophila cells, line 75E7 (one petri dish per point), were pretreated with actinomycin D (0.1 μg/ml) 30 min before infection and processed as in Fig. 1 (○); the drug was maintained at the same concentration throughout the experiment. For comparison, chicken embryo cells (■) and Drosophila cells (●) were infected and treated in the same way but without actinomycin D, at 30 and 25°C, respectively. To compare the VSV growth in each type of cell, the VSV production was expressed in PFU released per cell during 2-h intervals.*

synthesis was inhibited; protein synthesis lasted for a longer period and was reduced to 50% of its initial level after 4 to 6 h in the presence of

inhibitor. Nevertheless, after 9 h, the cells left the monolayer, and the number of viable cells decreased rapidly (after 13 h of treatment, cells still adhering to the Falcon flask represented only 10 to 20% of the cells treated at zero time).

Synthesis of VSV polypeptides in *Drosophila* cells. To determine the effect of VSV infection on host protein synthesis and to follow the effect of the cellular control on virus-specific protein synthesis, the intracellular proteins present during the virus cycle were examined.

Drosophila cells were pulse-labeled with ^{14}C-amino acids for 30 min at various times after infection. All the intracellular proteins were analyzed by electrophoresis in sodium dodecyl sulfate–5 to 13% polyacrylamide slab gels (Fig. 3). The infection led neither to modification nor to decrease of cellular protein synthesis, when compared to that of uninfected cells, yet virus-specific polypeptides such as N, NS, and M could be readily detected just after 1 h postinfection. The amount of polypeptide G was always very low, and L protein was obscure. Between 5 and 7 h, the amount of the virus structural proteins within the cells was the highest; then it started dropping, reaching a constant level at 13 h postinfection.

When the cells were pretreated with actinomycin D (0.1 μg/ml, 30 min before infection), the viral protein synthesis (including G and its partially glycosylated precursor, G_1 [45]) was much more important than in the absence of inhibitor (Fig. 4), and even L protein was visible. The cellular protein synthesis was progressively reduced after actinomycin D treatment, but after 9 h some of the host proteins remained, whereas in chicken embryo cells they were almost completely inhibited by the virus alone, 3 or 5 h after infection (Fig. 5).

However, one or even two new polypeptides (X) of approximately 20,000 molecular weight were present in infected *Drosophila* cells at 3 and 5 h postinfection. When the cells were pretreated with actinomycin D, these proteins were in greater amount and visible for a longer period of time (from 3 to 9 h). In infected chicken embryo cells, they also appeared simultaneously

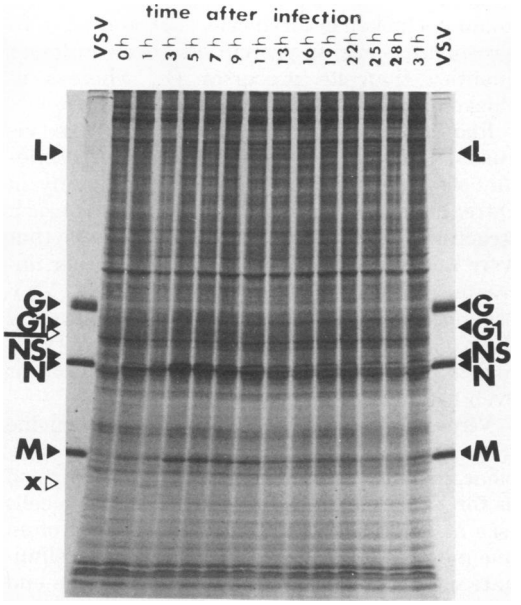

FIG. 3. *Protein synthesis in VSV-infected Drosophila cells. Drosophila cells, line 75E7 (one petri dish per point), were infected as described in Fig. 1. Uninfected cells (zero time) and VSV-infected cells (at the times indicated after infection) were pulse-labeled for 30 min with ^{14}C-amino acids as described in the text. The cells were collected, washed, disrupted, subjected to electrophoresis on a polyacrylamide slab gel, and processed for autoradiography. The long arrow indicates the standard cellular protein P50.*

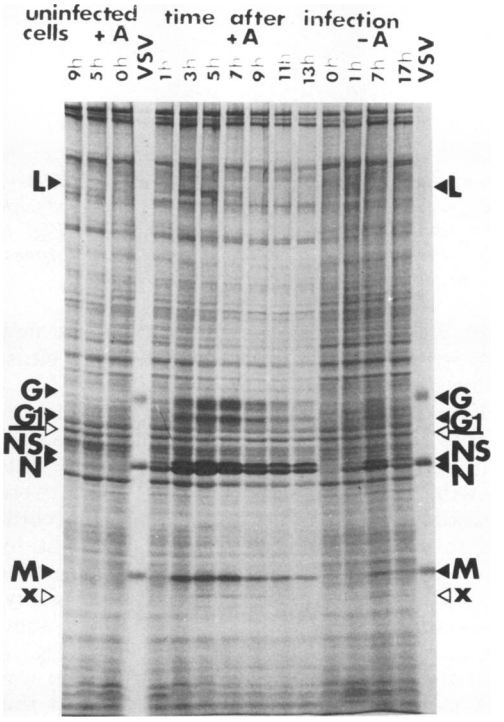

FIG. 4. *Protein synthesis in VSV-infected Drosophila cells treated with actinomycin D. Drosophila cells, line 75E7, were pretreated with actinomycin D (0.1 μg/ml) 30 min before infection and then infected as in Fig. 2. They were pulse-labeled with ^{14}C-amino acids and processed as described in the text. Control experiments were carried out on uninfected cells treated for various times with actinomycin D (left) and VSV-infected Drosophila cells that were not pretreated with actinomycin D (right).*

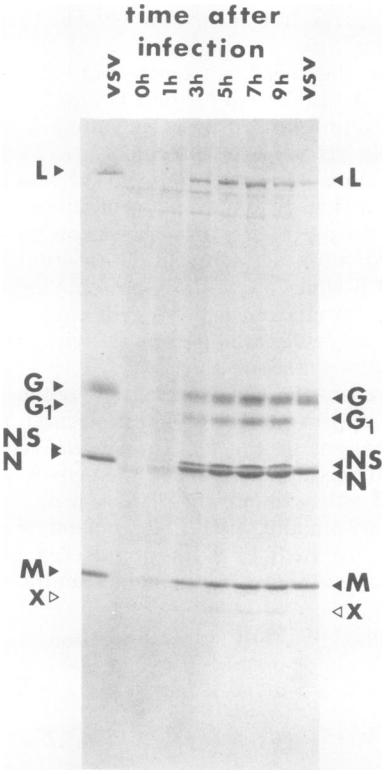

FIG. 5. *Protein synthesis in VSV-infected chicken embryo cells. Chicken embryo cells were infected with VSV as described for Drosophila cells in Fig. 1, labeled with ^{14}C-amino acids at the indicated times, and processed as described in Fig. 3.*

with the viral proteins. Therefore, these new polypeptides may be either virus-coded or virus-induced cell proteins.

Kinetics of viral protein synthesis in infected cells. To follow the amounts of the viral proteins synthesized in the cells during virus growth, autoradiograms (Fig. 3, 4, and 5) were quantified; molar ratios were evaluated according to each polypeptide molecular weight by scanning and measurement of the area under each viral protein peak. It was also necessary, however, to normalize these reports for a standard amount of cells. It was observed (Fig. 3) that the synthesis of each cellular protein was not modified after infection by VSV, so this normalization could be easily done by bringing back the value obtained to a constant amount of one cellular protein. In the case of actinomycin D-treated cells, only a very few cellular proteins were still synthesized in a constant amount after 9 h of treatment (Fig. 4); the other proteins had completely disappeared. One of the stable cellular proteins, P50 (50,000 molecular weight), was chosen as a standard.

The kinetics of viral protein synthesis in untreated or actinomycin D-treated cells are shown in Fig. 6 (note the different scales used). In untreated *Drosophila* cells, the amount of each viral protein increased for up to 4 to 5 h for the N protein and up to 5 to 7 h for the others, preceding the maximum level of virus release in the culture medium (maximum at 8 h). In chicken embryo cells, the N and NS protein peaks preceded those of the other viral proteins. In *Drosophila* cells, in the presence of actinomycin D, the times needed to reach the optimum values for each viral protein and for the virus release were similar. In the latter case, the shift down after 6 h was very probably due to cellular death. The synthesis of all the viral specific proteins was enhanced: 4 to 5 times more N, NS, and M, and 14 times more G and G_1, than in untreated infected cells.

To compare the relative yields of G and G_1 in treated or untreated *Drosophila* cells and in chicken embryo fibroblasts, the sum G + G_1 was calculated and compared to M protein amount. In *Drosophila* cells, the ratio (G + G_1)/M was 0.45 and 0.37 at 5 and 7 h, respectively; in actinomycin D-pretreated cells, these values increased to 1.32 and 1.35, close to the values found in chicken embryo cells (1.38 and 1.29). In *Drosophila* cells, G protein was even in lesser quantity than its precursor G_1, whereas in chicken embryo cells it was the reverse.

Protein composition of the mature viruses. The deficiency in G protein in *Drosophila* cells and its enhancement after actinomycin D treatment led us to study the amounts of each structural protein in the mature viruses that were released from *Drosophila* cells, either untreated or treated with actinomycin D. VSV production increase could be explained either by the release of more infectious virus particles with higher G content, by virus overproduction, or even by both mechanisms.

Virus proteins were labeled with [^{14}C]leucine from 1 to 24 h after infection in a leucine-deficient medium. Virus purification was performed as for VSV released from chicken embryo cells (see Materials and Methods), except that often one more sucrose gradient was needed to eliminate cellular protein contaminants. At the end of the purification process, the amount of prepared virus was estimated by optical density measurement and protein concentration determination; actinomycin D-treated culture yielded 5 to 10 times more purified virions than did untreated *Drosophila* cells.

The proteins of the purified viruses were analyzed by electrophoresis on polyacrylamide slab gels and by autoradiography (Fig. 7); the amount of G protein in the viruses released from *Dro-*

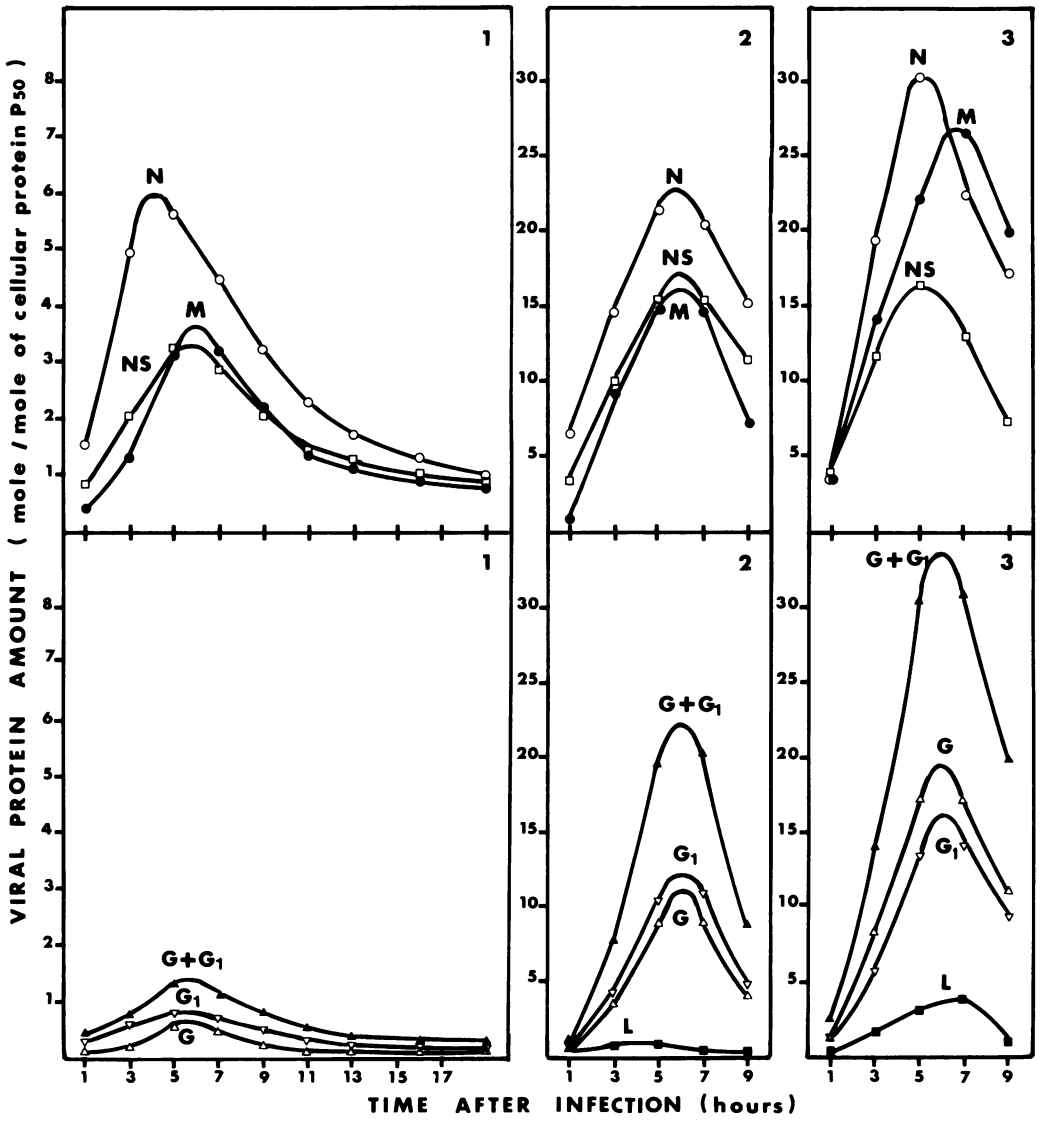

FIG. 6. *Kinetics of VSV polypeptide synthesis in actinomycin D-treated or untreated Drosophila cells and in chicken embryo cells. Autoradiograms of Fig. 3, 4, and 5 were scanned, and the amount of each viral polypeptide was calculated. For VSV grown in Drosophila cells, these values were brought back to a constant amount of a cellular protein (P50) as explained in the text. For VSV grown in chicken embryo cells, units were arbitrarily chosen because all the cellular protein synthesis declined after infection. (1) Drosophila cells; (2) Drosophila cells treated with 0.1 μg of actinomycin D per ml; (3) chicken embryo cells. Proteins N (○), NS (□), M (●), G (△), G_1 (▽), G + G_1 (▲), and L (■).*

sophila cells, either untreated or treated with actinomycin D, was clearly lower than in the viruses released from chicken embryo cells (according to both intensity of staining with Coomassie blue and to incorporation of [^{14}C]leucine). Moreover, it migrated faster. The molecular weight of each viral structural protein was determined by comigration using the polypeptide subunits of yeast RNA polymerases A and B as markers (4, 13, 14). The average of eight determinations for structural proteins of VSV released from chicken embryo cells gave 210,000 molecular weight for L protein, 64,000 for G, 45,000 for N, and 29,000 for M. The G protein of the viruses released from *Drosophila* cells had a molecular weight of about 62,000, which could be due to differences in glycosylation and sialylation (45).

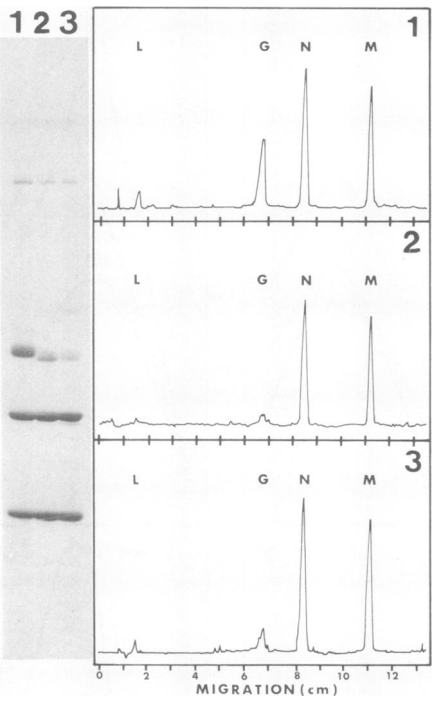

FIG. 7. *Protein content of mature viruses released from untreated and actinomycin D-treated Drosophila cells. VSV was grown and labeled on chicken embryo cells (1) or Drosophila cells (2 and 3) either untreated (2) or treated with 0.1 μg of actinomycin D per ml (3) as described in the text. After 24 h of multiplication at 30 or 25°C, viruses were collected, purified, and analyzed by electrophoresis on a polyacrylamide slab gel. Left, Virus polypeptides stained with Coomassie brilliant blue. Right, Scannings of the autoradiograms.*

The stoichiometric amount of each viral protein was calculated after scanning the gel, by measurement of the area under each protein peak, taking into account their molecular weight as estimated above. Whereas VSV grown in chicken embryo cells contained 0.53 G molecule for 1 of M, the virus released from *Drosophila* cells, either pretreated with actinomycin D or untreated, was poorer in G protein: only 0.12 and 0.13 G molecule for 1 of M, respectively (Table 2). The same result was obtained even when the virus was collected at different stages of the virus cycle, during either the phase of intensive production or the carrier state. This finding was extended to another virus strain, VSV Indiana (standard), to another virus serotype, VSV New Jersey, and to another *Drosophila* cell line, line 75B (Table 2). Whatever the VSV strain or serotype used for infection and whatever the *Drosophila* cell line used as host, the released virus always contained four to five times less G than its counterpart produced by chicken embryo cells. Electron microscopy observations showed that the virions released from *Drosophila* cells did not contain as many spikes as viruses grown in chicken embryo cells (Fig. 8). Particles surrounded with very few spikes were observed (Fig. 8, B-3), as well as others that were more regularly coated (Fig. 8, B-1 and B-2).

Since G protein forms the more external structure of the virus (the spikes), these results could reflect a more important weakness during purification of the virus produced by *Drosophila* cells. Electron microscopy observations (results not shown) indicated that there was no clear difference between purified viruses and those collected just before observation (8 h after *Drosophila* cell infection, either untreated or treated with actinomycin D). Broken particles were indeed present in the purified virus preparations, but they contained as many spikes as unbroken particles.

Proteolytic activity, which could be responsible for this protein deficiency, was investigated. Incubations for 24 h at 25°C of purified VSV released from chicken embryo or *Drosophila* cells, in the presence of an infected *Drosophila* culture medium or in the presence of an infected *Drosophila* cell extract (cells lysed in hypotonic medium and sonicated), revealed neither a high level of proteolytic activity, which would deteriorate the whole virus, nor a specific one, which would preferentially degrade the G protein (data not shown).

DISCUSSION

It has been found that VSV establishes a persistent noncytopathic infection in cultured *Drosophila* cells. In the present study, no inhibition of cellular protein synthesis occurred even during the first hours, when all cells were initially infected by VSV and when they actively produced viruses. When the virus production dropped to the carrier state plateau, no modification of the cellular protein pattern was observed, and involvement of DI particle synthesis seemed unlikely; indeed, such particles were not detected during the first hours after infection in experimental conditions of one-step infection. Similar findings have been obtained with Sindbis virus grown in *Aedes albopictus* (24). This does not exclude the possibilities that DI particles or short VSV-specific RNA could be produced later on in persistently infected cells, as suggested by the results of Eaton (16), who found short Sindbis virus-specific RNA in persistently infected mosquito cells.

During the VSV growth cycle in *Drosophila*

TABLE 2. *Effect of host cells on polypeptide composition of various VSV strains*

Virus strains[a]	Host cells[a]	No. of expts	Protein composition of the released virus[b]			
			M	N	G	L
VSV Indiana BT78 1555	Chicken embryo	12	100	125	53	2.5
	Drosophila (75E7)	11	100	113	12	0.75
	Drosophila (75B)	5	100	140	11	—
VSV Indiana (standard)	Chicken embryo	3	100	131	55	2.8
	Drosophila (75E7)	4	100	153	13	0.7
	Drosophila (75B)	2	100	116	15.5	—
VSV New Jersey	Chicken embryo	2	100	153	53	5
	Drosophila (75E7)	2	100	210	13	—
	Drosophila (75B)	2	100	145	15.5	—
VSV Indiana BT78 1555	*Drosophila* (75E7) + actinomycin D	17	100	99	13	0.75

[a] VSV strains were grown and labeled with [^{14}C]leucine on chicken embryo or *Drosophila* cells, line 75E7 or 75B, as described in the text.

[b] After virus purification, the viral polypeptide chains were separated on a polyacrylamide slab gel and their molecular weights were determined as described in Fig. 8. Autoradiograms were scanned, and the molar ratio of each polypeptide was calculated and normalized to 100 equivalents of protein M.

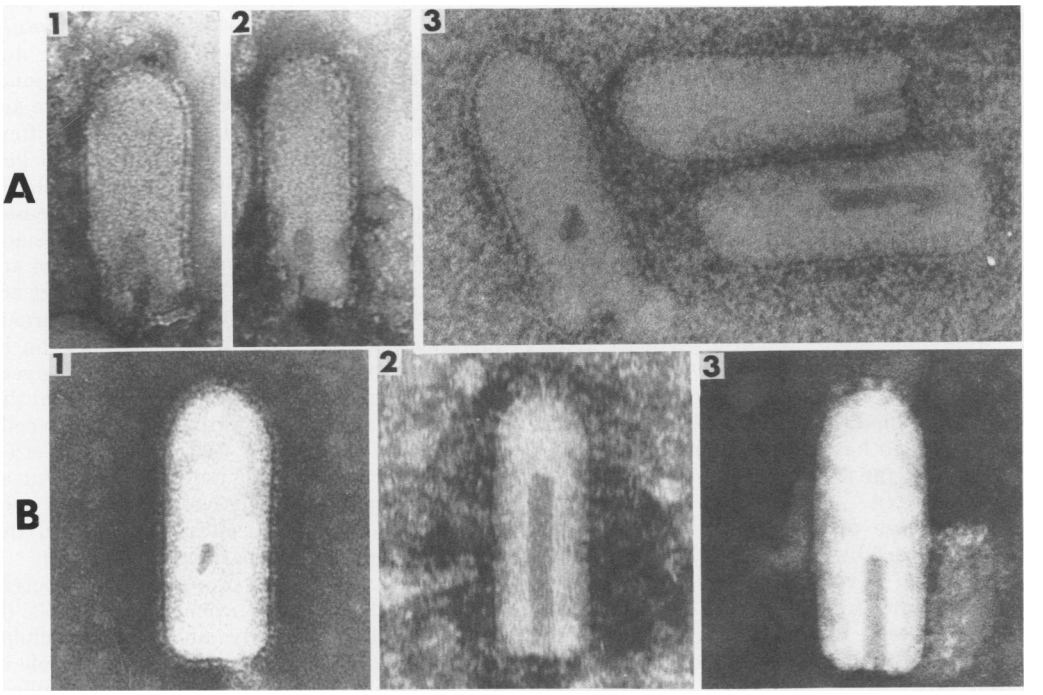

FIG. 8. *Electron micrographs of VSV released from chicken embryo and Drosophila cells. Chicken embryo or Drosophila cells were infected with VSV as described in the text. The culture media were collected 8 h after infection and clarified by low-speed centrifugation (15 min at 5,000 rpm). Viruses were pelleted through a 20% sucrose cushion (2 h at 30,000 rpm in an SW41 rotor with a Beckman ultracentrifuge). The pellets were suspended in LSB. Droplets of unfixed viral suspension were immediately negatively stained with 2% sodium tungstate. (A) Viruses released from chicken embryo cells; (B) viruses released from Drosophila cells. Magnification: A-1, A-2, and B-2, ×190,000; A-3, B-1, and B-3, ×230,000.*

cells, each viral protein appeared in the cells, reaching a maximum level between 5 and 7 h after infection; then their synthesis declined to a constant rate which was maintained during the carrier state. L polypeptide, which is implicated in virus transcription and replication (18), was obscured by cellular proteins of closely related molecular weight, perhaps due to its low level.

The glycoprotein G, which forms the surface projections on the virus (9, 47), was always poorly represented compared to the other virus proteins, and its electrophoretic mobility was somewhat modified. This result has been previously mentioned by Mudd et al. (34), who noticed that the G protein of a VSV mutant selected on *D. melanogaster* cells was reduced in amount as compared to the ratio usually found in vertebrate-grown VSV. This could be due to a cellular control of gene expression at level of G mRNA transcription, G protein traduction, or maturation. The molar ratios of the various VSV mRNA's will have to be compared with their protein products to determine whether this cellular control acts at or after transcription level.

In an attempt to understand the nature of this control, experiments with actinomycin D were carried out. The control process was disturbed by the drug, and VSV multiplication was considerably increased. This result is not in agreement with that of Artsob and Spence (2), who observed an enhancement of VSV production by actinomycin D in persistently infected cells, but not in freshly infected cells. Unfortunately, we cannot conclude that the virus in the presence of actinomycin D became cytolytic, since the cells were damaged by the drug presence alone. Nevertheless, the actinomycin D effect was obtained at a very low concentration which is not thought to impair cellular mRNA synthesis; therefore it can hardly be explained by competition for the translational step between cellular and viral mRNA.

This increase of VSV production could be explained either by virus overproduction or by the release of G-rich virus particles of higher infectivity, since infectivity and G protein content seemed to be related (12, 25). In fact, more virions were purified from actinomycin D-treated cell culture, and these viral particles were also deficient in G protein.

At the intracellular level, actinomycin D increased the synthesis of each viral protein, but, surprisingly, G-protein synthesis was more enhanced than that of the others. This peculiar effect of the drug on G synthesis may be the consequence of G transduction localization. G is the only viral protein synthesized on membrane-bound polyribosomes; it cannot be excluded that the drug acts on a posttranscriptional event and alters membrane-bound polyribosomes and free polysomes in a different way. It is also possible, however, that actinomycin D disturbs a hypothetical specific cellular control acting on G synthesis.

The fact that viruses produced by actinomycin D-treated cells did not contain a standard proportion of G, whereas the intracellular yield of $G + G_1$ was high, suggests that G synthesis rate is not the only limiting factor controlling VSV production. It seems likely that G proteins synthesized in *Drosophila* cells have some host-dependent defect which prevents their incorporation in the cellular membrane, the virus envelope, or both. This defect could appear during the G-protein maturation process, which requires cellular enzymes for glycosylation and sialylation (5, 33, 41). This assumption is favored by three observations: (i) viruses grown in *Drosophila* (J. Laurent, personal communication) and mosquito cells (41) were unsialylated; (ii) the difference in mobility between the G proteins of the viruses released from *Drosophila* and chicken embryo cells (Fig. 7) could be due to either this absence of sialic acid or additional changes in the carbohydrate chain; (iii) the antigenicity of VSV adapted to *Drosophila* differs from that of the initial VSV grown in chicken embryo cells (28). These defects could be related to the poor G content of the viruses released from *Drosophila* cells, since variations of sialic acid content and carbohydrate composition are associated with changes of hemagglutinating activity and infectivity (1, 25, 33) and since various modifications of G impair its incorporation in the viral envelope. Indeed, incompletely glycosylated G polypeptides were not detected in the mature viruses released from vertebrate cells (23). VSV glycoproteins that were synthesized in cells infected by *tsO45* mutants (complementation group V) at restrictive temperature were found neither on the cell surface nor in the noninfectious released particles (12, 27, 42).

Finally, two different properties of G protein were recently suggested in mammalian cells: (i) its ability to cause early inhibition of cellular DNA and RNA synthesis (32); and (ii) a role in the equilibrium between viral genome replication and transcription (30). If this were also the case for *Drosophila* cells, G-protein synthesis control could be a way for insect cells to prevent inhibition of their own macromolecular synthesis and to counteract viral replication.

ACKNOWLEDGMENTS

We are grateful to every one who contributed to this work in the laboratory, especially to F. Diatta and B. Simonet for

providing *Drosophila* cells and chicken embryo fibroblasts. We thank F. Bussereau, D. Contamine, G. Brun, P. Printz, and A. Combard for fruitful discussions and critical reading of the manuscript.

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